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THE EXCRETION OF PROGESTERONE

HUGH STANSFIELD

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Thesis
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THE EXCRETION OF PROGESTERONE

Hugh Stansfield, B. Sc.

A thesis submitted from the Department of Physiology and Pharmacology in fulfillment of the requirements for the degree of Master of Science at the University of Alberta.

1939.

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STATEMENT OF PROBLEM

Many physiological processes are under chemical control. The chemical controlling agents are called hormones, and are produced in the endocrine or ductless glands. The pituitary body, and the gonads are the chief sources of sex hormones, which control reproductive processes, and secondary sex characteristics. The hormones act like catalysts, and are usually not destroyed during their action. Sex hormones are usually excreted by the kidneys, and some may be recovered from the urine, unchanged, or in some simple combination such as the glucuronic acid ester. Progesterone is one of the female sex hormones. Unlike some of the others, it is changed to another compound, pregnanediol, before excretion. It has recently been shown that pregnanediol is excreted as sodium pregnanediol glucuronidate, and a method has been published for the quantitative determination of this compound in human urine. The present paper is an attempt to extend this study to the urine of other animals with human urine, as a useful standard control. As progesterone is essential

to maintain normal pregnancy, the importance of this study may readily be imagined. However this aspect will be discussed more fully later on. A brief introduction will now be given to show the relation of the present work to the larger field of research on the sex hormones.

INTRODUCTION

The female sex gland is the ovary, which at birth already contains all the potential germ cells in its outer or cortical layer. (Fig. 1.) These cells are at the primary oocyte stage of development. The oocyte together with the single layer of germinal epithelial cells, stratum granulosum, by which it is surrounded, forms the primary ovarian follicle. There is no change from birth until puberty.

At puberty, one or two of the primary follicles will commence to grow. The stratum granulosum proliferates rapidly. Soon a cavity appears in the stratum granulosum, and the vesicular stage has begun. As the follicle continues to grow, the cavity becomes the most notable feature, with the oocyte at one side surrounded by cells of the stratum granulosum. (Fig. 2.) The cavity is filled with liquid called the liquor folliculi. When the follicle is "ripe", it ruptures, and the oocyte which is by now almost a mature ovum, is swept out with the liquor folliculi into the uterine tube.

Figure 1.

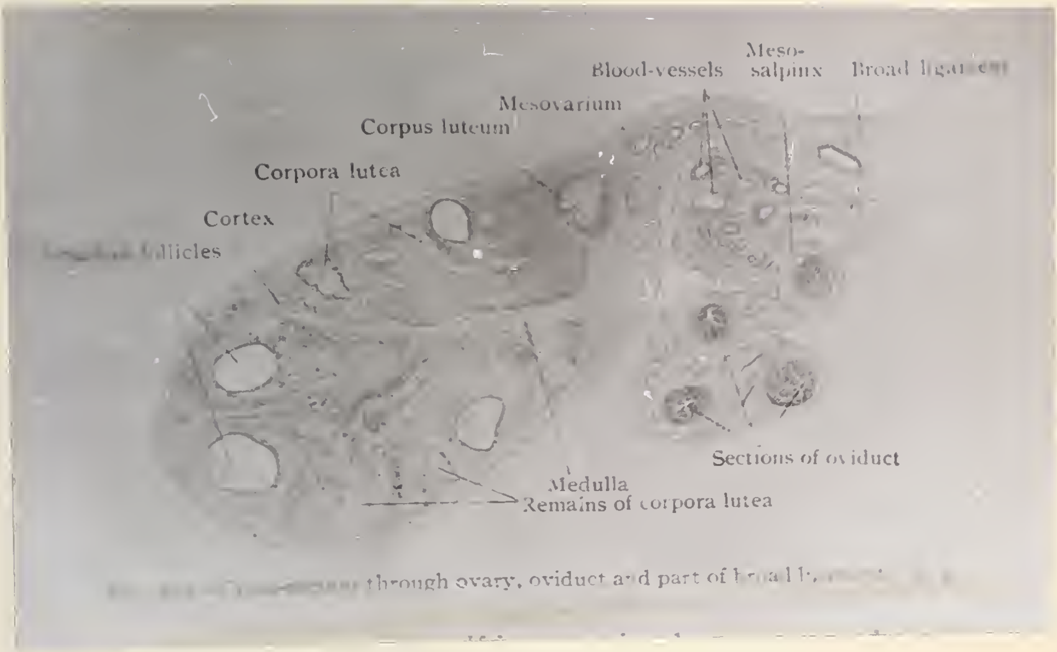
Cross-section through Ovary. X 5

(Piersoll's "Normal Histology.")

Figure 2.

Cross-section through Graafian follicle. X 90

(Piersoll's "Normal Histology.")



[illegible] through ovary, oviduct and part of broad l[illegible]

F I G. 1.

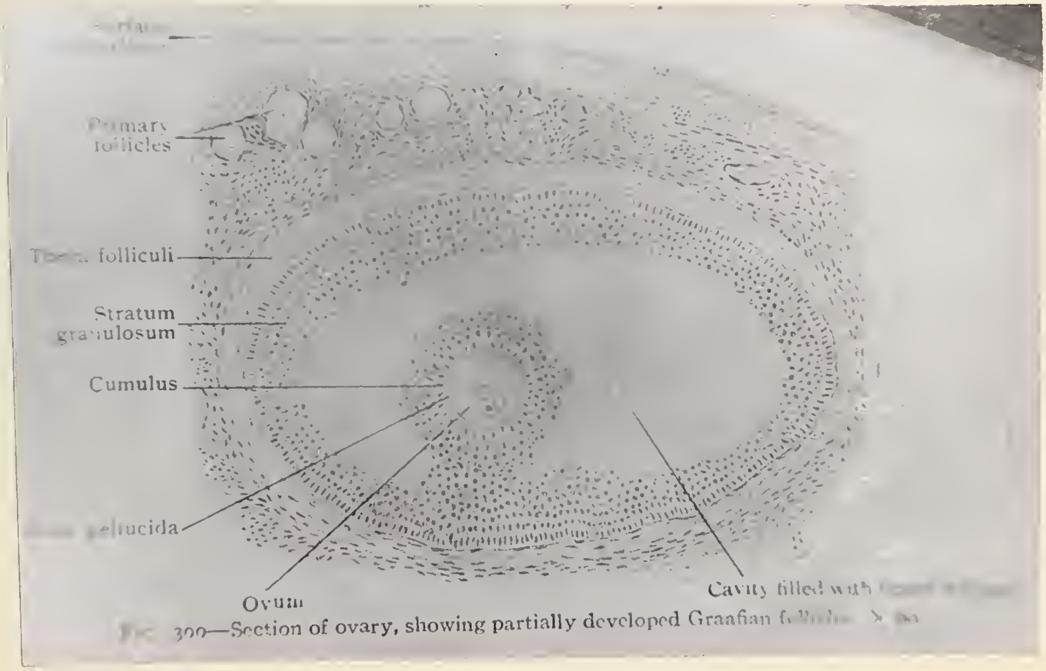


Fig. 300—Section of ovary, showing partially developed Graafian follicle. [illegible]

F I G. 2.

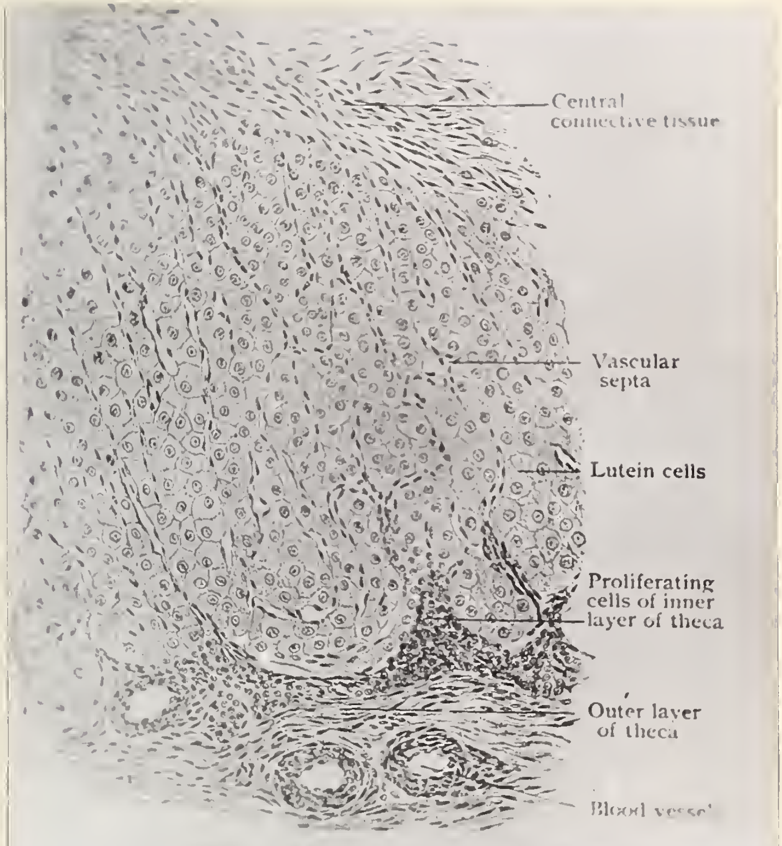
To return to the ruptured Graafian follicle. The extrusion of the ovum and liquor is followed by the rupture of some of the small blood vessels around the follicle. These vessels fill the empty follicle with blood, which clots to form the corpus haemorrhagicum. This clot is gradually replaced by the ingrowth of specialized lutein cells and connective tissue from the old stratum granulosum. When this process, luteinization, is complete, the resulting structure is called the corpus luteum or yellow body. (Fig. 3.) The corpus luteum undergoes gradual degeneration, unless impregnation has occurred, in which case it becomes larger and persists during pregnancy. As the structure atrophies, the lutein cells are partially replaced by scar tissue, and the resulting structure is called the corpus albicans or white body. It gradually disappears, and is of no further importance.

The series of events just outlined for the ovary is accompanied by equally definite structural changes in the uterus and vagina, particularly the inner glandular layer, the mucosa or endometrium. The period during which the follicle is growing is marked by a rapid growth in the endometrium.

Figure 3.

Section through human corpus luteum X 80

(Piersoll's "Normal Histology.")


Central connective tissue
Vascular septa
Lutein cells
Proliferating cells of inner layer of theca
Outer layer of theca
Blood vessel

Section of young corpus luteum × 71

F I G. 3.

At the time of ovulation, the growth stops, and no further change is noted until the corpus luteum develops.

At this time, the uterine glands, which were previously straight or only slightly coiled tubes, become tortuously coiled and dilated. If fertilization does not occur, the corpus luteum degenerates, and the endometrium simultaneously undergoes degeneration with haemorrhage, and loss of the superficial part of the mucosa.

The series of events just described, with humans recurs regularly in periods of approximately 28 days. This is the menstrual cycle, and is analagous to the estrous cycle in lower animals.

The corpus luteum phase is sometimes referred to as pseudopregnancy. If fertilization occurs, it persists as real pregnancy. The uterus at this stage, is in suitable condition for the implantation of the fertilized ovum.

While the events of the menstrual cycle, and pregnancy, have been known for a long time; it is only within the last ten to fifteen years, that an understanding of the mechanism controlling the cycle has been gained.

Experiments involving removal, transplanting, and grafting, carried out during the last twenty five years or so, have given a fairly clear idea of the manner in which the various endocrine glands and hormones control reproductive phenomena. Later, deductions made as to the function of a gland, from surgical procedures, are verified by the preparation of active extracts of the gland. The ability of such extracts to remove deficiency symptoms, and maintain normal function is considered proof of the deductions. The final stage is then to isolate , purify, and identify chemically, the active principles of such extracts. (1.)

The anterior lobe of the pituitary gland seems to be the central control station. Concentrated extracts are known, but the active principles have not yet been isolated. It is a hormonal stimulus from the pituitary that ~~the~~ starts the Graafian follicle ripening. The follicle produces a hormone, estrin, which was independently isolated in pure crystalline form in 1930 by several different workers, Doisy, Marrian, and Butenandt. Estrin is actually two compounds with the same action but slightly different structure. It is this hormone, which brings on the

typical estrus response in the uro-genital tract. The further progestational development of the mucosa is due to the corpus luteum hormone, progesterone, isolated in 1934 by a number of different workers, and synthesized in the same year. Progesterone also maintains the uterine mucosa throughout pregnancy. In Fig. 4 will be seen a diagrammatic correlation of hormone activities as shown in the Parke-Davis Therapeutic Notes of April 1935. It has been stated (2) that the cyclic activity is due to a reciprocal action of ovary and anterior pituitary on each other. The corpus luteum requires estrin both to become and to remain functional.

Histologically speaking, the corpus luteum is a very prominent structure, so that it is not surprising that there has long been speculation as to its function. In 1901, Fraenkel (1, 3.), at the suggestion of the German enbryologist Born, was able to terminate pregnancy in rabbits by removing the corpus luteum. Between 1911 and 1920, it became fairly well-established that the presence of an active corpus luteum inhibited ovulation. Since Fraenkel, there have been many more

Figure 4.

Interrelations of the various endocrine glands.

(Parke-Davis: Therapeutic Notes, April 1935.)

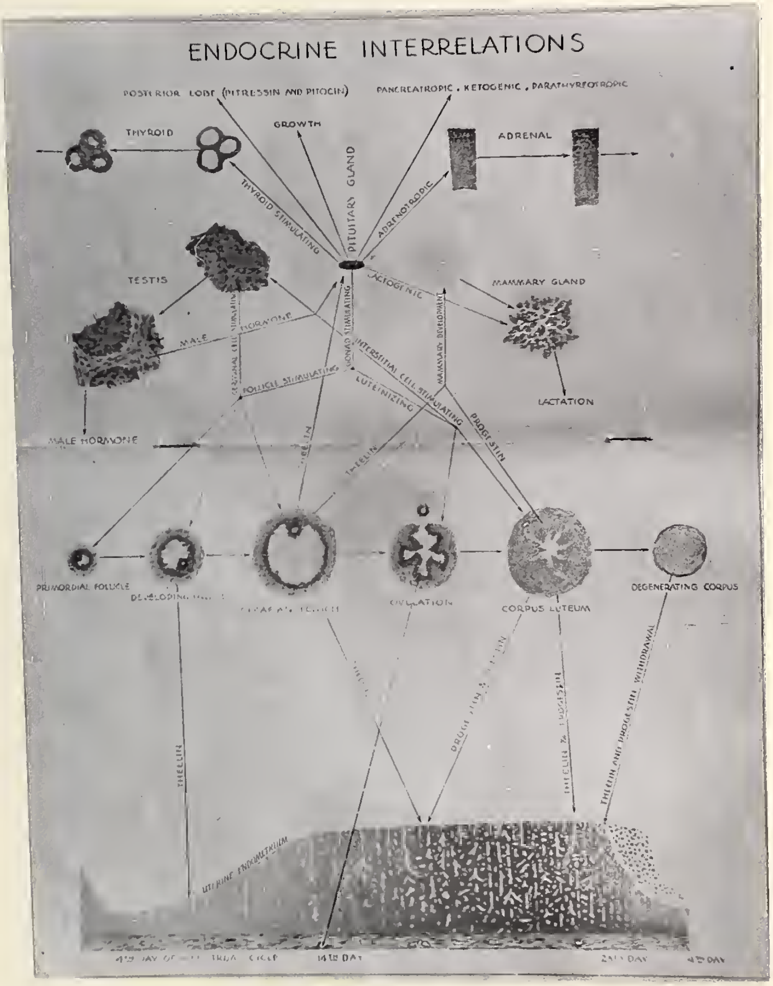

ENDOCRINE INTERRELATIONS
POSTERIOR LOBE (PITRESSIN AND PITOCIN)
PANCREATROPIC, KETOGENIC, PARATHYREOTROPIC
THYROID
GROWTH
PITUITARY GLAND
ADRENAL
THYROID STIMULATING
ADRENOTROPIC
TESTIS
LACTOGENIC
MAMMARY GLAND
MALE HORMONE
GERMINAL CELL STIMULATING
GONAD STIMULATING
INTERSTITIAL CELL STIMULATING
MAMMARY DEVELOPMENT
FOLLICLE STIMULATING
LUTEINIZING
LACTATION
PROGESTIN
MALE HORMONE
THEELIN
THEELIN
PRIMORDIAL FOLLICLE
DEVELOPING FOLLICLE
GRAAFIAN FOLLICLE
OVULATION
CORPUS LUTEUM
DEGENERATING CORPUS
THEELIN
THEELIN
PROGESTIN & THEELIN
THEELIN & PROGESTIN
THEELIN AND PROGESTIN WITHDRAWAL
UTERINE ENDOMETRIUM
4th DAY OF MENSTRUAL CYCLE
14th DAY
28th DAY
4th DAY

FIG. 4.

experiments on the removal of the corpora lutea during pregnancy. The results have appeared inconsistent and led to confusion, due probably to progesterone or some equivalent being formed in the placenta (2, 4) or the cortex of the adrenal. Despite the confusion about the necessity of the corpus luteum during all of pregnancy, it has been generally accepted since about 1907, that the corpus luteum is necessary to prepare the uterus for the implantation of the fertilized ovum. Progesterone or some related substance is necessary for the maintenance of pregnancy.

Active extracts of corpora lutea have been used since 1914. As the distinction between estrin and progesterone was unknown at that time, and most extracts contained both, there was confusion in the tests used to assay the potency of extracts. By 1930 pure preparations of estrin were available, and in that same year, Hisaw et al., and Corner and Allen, obtained fairly pure active preparations of progesterone.

Corner and Allen (1, 3) in 1929 were the first to prove definitely that progestational changes were due to the corpus luteum hormone. The next year,

Hisaw and Leonard showed that it was a "one-two" reaction, needing estrin to start the development, followed by progesterone. In Fig. 5 and Fig. 6., will be seen sections of the uteri of the adult castrate monkeys. The monkey represented in Fig. 5 was given injections of estrin alone. The picture is typical of an estrus uterus. The monkey represented in Fig. 6 was given estrin followed by progesterone. Fig. 6 shows a perfectly normal pseudopregnant or pre-menstrual uterus.

To explain the apparent continuation of corpus luteum function after the removal of corpora lutea it has been necessary to find some other possible source of progesterone. While still a moot point, it seems generally accepted at the present time that progesterone and estrin are formed in the placenta during the later part of pregnancy. (2,4.)

The other source suggested earlier, the cortex of the adrenal gland, has not been suggested directly in the literature, but appears possible from certain articles published recently. (13) Crystalline progesterone will keep alive adrenalectomized ferrets (14) and rats (15), due apparently to a close chemical affinity between progesterone

Figure 5.

Endometrium of an adult monkey, which was castrated and given subcutaneous injections of estrin for an extended period.

(W. M. Allen)

Figure 6.

Endometrium of an adult castrate monkey which was first given estrin, followed by progesterone treatment.

(W. M. Allen)

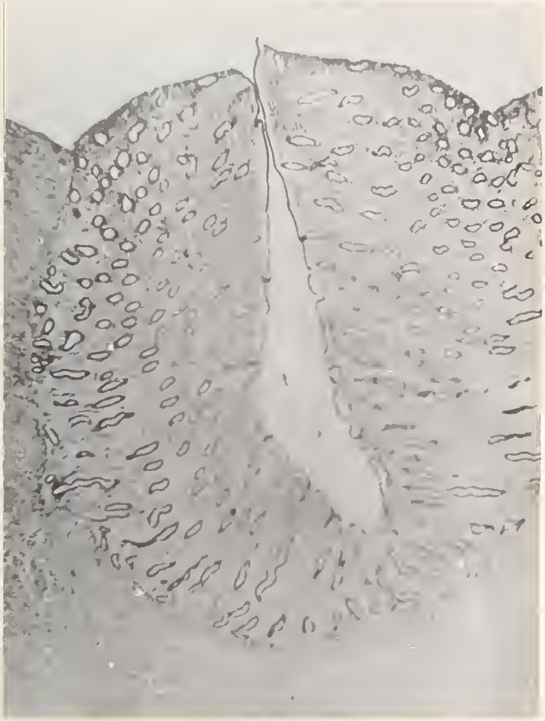


FIG. 5.

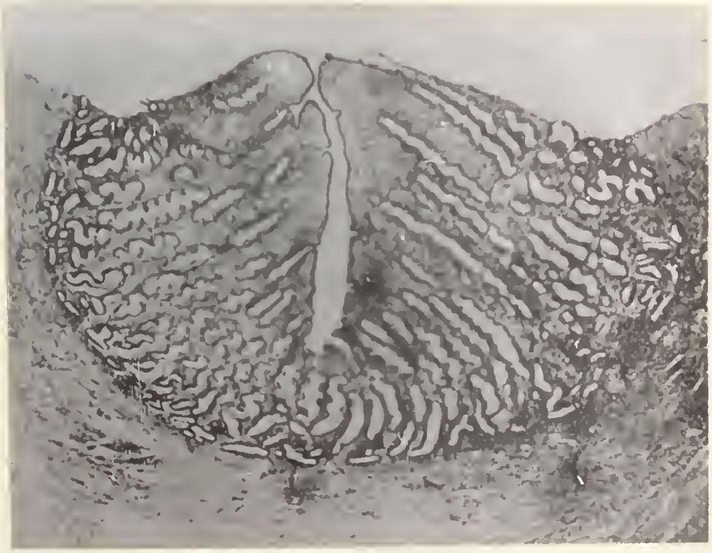


FIG. 6.

and various cortical sterols. Finally a synthetic preparation, desoxy-corticosterone, even more closely resembling progesterone has been used to treat toxaemias of pregnancy. Lack of progesterone can cause similar conditions, and treatment with progesterone has been used.

The rapidity with which endocrine research is progressing at the present time, can be shown dramatically by the following facts. One volume of Die Berichte, 67 B, contains:

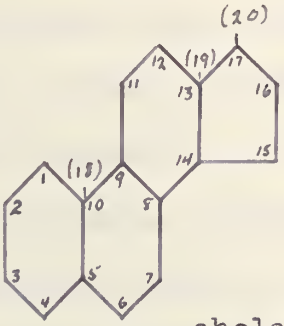
1. A report of two of the first four independent isolations of pure progesterone.
2. The first suggestion of the correct structure.
3. Three syntheses of the substance.

Isolation of pure progesterone was reported first by Butenandt (5.) and Slotta (6.), later by Allen (7.) and Hartmann (8.). Slotta and Fels (9.) soon after suggested the correct formula, which was confirmed by synthesis by Butenandt et al. (10, 12.) and Fernholz. (11.)

The reduced form of progesterone, pregnanediol found in pregnancy urine, was first described in 1929 by G. F. Marrian (16.). In 1931, Butenandt

suggested an incorrect formula (17.). In 1932, Bernal (18.), Marrian (19.), and others worked out the correct formula. Butenandt and Schmidt in 1934, used pregnanediol as a starting point for a synthesis of progesterone by a mild oxidation with chromic acid (12.)

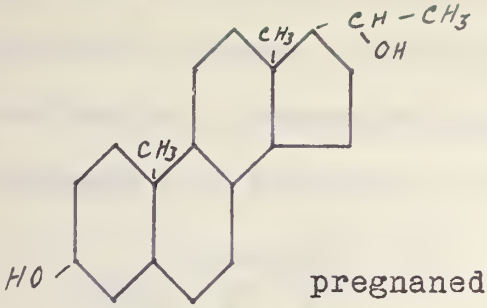
Before proceeding further, it seems desirable to discuss the structure of some of the hormones, we have discussed so far. They all belong to the chemical group known as the sterols, all possessing a four ring nucleus, known as the cholane nucleus. Gilman (13) prefers cyclopentanoperhydrophenanthrene as the basic structure.



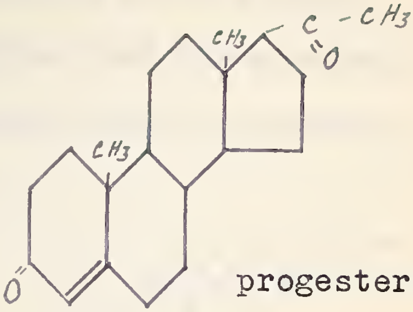
cholane nucleus.

Pregnanediol and progesterone are named as derivatives of a hydrocarbon 17-ethyletiocholane or pregnane. Therefore the correct chemical names for these compounds are 3, 20-pregnanediol, and Δ^4 pregnene 3, 20-dione. The space structures are as

as follows,



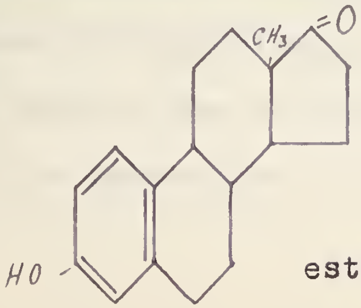
pregnanediol



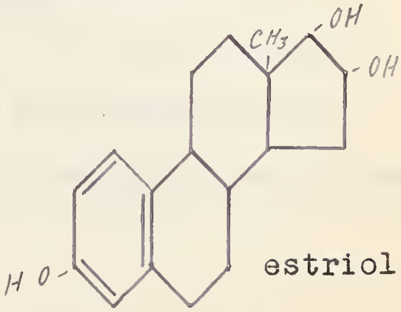
progesterone

In the formula for pregnanediol, it will be noted that there are nine centres of asymmetry at C-3, 5, 8, 9, 10, 13, 14, 17 and 20. From this there are theoretically 2^9 or 512 possible stereo-isomers. Fortunately, Nature is highly specific in her synthesis and only two naturally occurring pregnanediols are known, differing only in a cis-trans relationship between the C3-OH and the C5-H. Other isomers are known from in vitro syntheses, but are of importance only in theoretical chemistry.

The follicular hormone, estrin, is actually a mixture of two compounds, estriol and estrone, whose formulae are shown below. These compounds are also known as theelol, theelin, progynon etc.

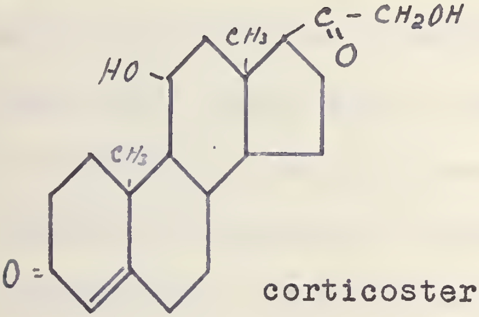


estrone

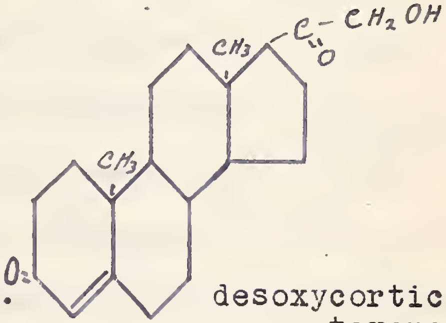


estriol

For comparison, the structure of an adrenal cortical hormone, corticosterone, and the synthetic derivative, desoxycorticosterone, used in treating toxaemias of pregnancy, follow,-



corticosterone.



desoxycorticosterone.

It is obvious, that when dealing with such complex mixtures as tissue extracts, and urine, no no research can progress very far, or very rapidly, without satisfactory methods of analysis. The four separate isolations of progesterone reported in 1934, were the direct result of a satisfactory method of biological assay, developed by Corner and Allen (20) in 1930. Dr. M. K. McPhail, formerly of this University, developed an improved method for the biological assay of progesterone (21). As no progesterone is excreted in the urine, as such, it is obvious that these methods are of little use in clinical work, where the production of progesterone must be followed

over a period of time, and without tampering with the patient.

Pregnanediol was discovered accidentally during an investigation on estrin (16.) This was fortunate as it is completely inactive biologically, so that there would have been no way to detect its presence biologically, to prove any hypothesis that it (PROGESTERONE) was excreted through the kidneys. However, at the time progesterone was isolated, the structure and chemistry of pregnanediol were already known. When the relation~~s~~ between them was understood, it stimulated research to find a satisfactory analytical procedure for estimating the pregnanediol concentration in urine.

The first, and so far the only satisfactory procedure for the estimation of pregnanediol, is that of Venning and Browne at McGill. (22.) This procedure has been used for a considerable part of the present investigation.

The Venning procedure isolates pure sodium pregnanediol glucuronidate. Weil (23.) reported that in some cases, no pregnanediol could be recovered in this form, but that a new procedure, to be published soon, could separate all the pregnanediol as the free alcohol. A request was made for

the details of his procedure, which he was good enough to furnish. This method was also used in the present work, but with less success.

METHODS

As the methods of Venning (22.) and Weil (private communication of April 29, 1938.) were used extensively throughout this investigation, it seems proper to give them in some detail.

Estimation of Na Pregnanediol Glucuronidate-- E. H. Venning.

Extract sample of urine four times with a total of one third its volume of n-butyl alcohol. For a sample of 1 litre, amounts used would be 150, 75, 50, and 50 cc. The BuOH extracts are centrifuged, and the clear supernatant liquid run into a distilling flask. The alcohol is evaporated to dryness under reduced pressure, and the residue taken up in 60 cc. N/10 NaOH. This is extracted four times with BuOH (20, 20, 10 and 10 cc.) and the BuOH extract washed twice with 5 cc. portions of water. The extract is centrifuged, if necessary, and evaporated to dryness under reduced pressure. To the residue in the flask, 5 cc. of water and 10 cc. of anhydrous acetone are added. Transfer to a small Erlenmeyer. Rinse distilling flask, and make up to 100 cc. with dry acetone. Leave over-night at 5-10° C. A fine white crystalline precipitate settles out. Decant clear super-

natant acetone and centrifuge remainder. Pour off acetone.

Add a few drops of water to centrifuge tube and dissolve precipitate in hot ethyl alcohol. Filter into a previously weighed beaker, and evaporate to dryness on a water bath. Weigh product thus obtained. Pure Na pregnanediol glucuronidate melts at 268-271° C with decomposition.

Total Pregnanediol in Human Urine--P.G. Weil.

Take an aliquot portion of a 24 hour specimen. Bring to pH7 and incubate at 37°C for 24 hours, or until it becomes pH8. Extract twice with a total of one quarter volume of n-butyl alcohol. For 300 cc. sample, use 50 and 25 cc. The BuOH extracts are centrifuged, and the clear supernatant layer is evaporated to dryness in a distilling flask. Residue taken up in 10 cc. acetone and 10 cc. N/10 NaOH. Warm until dissolved and transfer to a small Erlenmeyer. Rinse flask twice with 5 cc. hot water and use to wash precipitate. Precipitate transferred to original Erlenmeyer. Rinse flask with 30 cc. Warm N/10 NaOH and add to Erlenmeyer slowly. Cool overnight. Filter by suction. Rinse flask twice with

5 cc. hot water, and use to wash precipitate. Precipitate transferred to original Erlenmeyer, and dissolved in 5, 10 or 20 cc. of acetone, depending on amount of precipitate present. Heated to boiling to effect complete solution. Three volumes of N/10 NaOH are added with moderate heating and the mixture set aside at room temperature until precipitate appears, and then placed in cold overnight. Filter and wash once with 5 cc. hot water. Dissolve precipitate in minimum amount of hot ethyl alcohol-usually 5 to 10 cc. Two volumes of water added with moderate heating. Allow to precipitate as before and transfer precipitate collected by suction filtration back to flask. Dissolve in hot ethyl alcohol, and filter by suction into a weighed vessel. Evaporate off EtOH and weigh pregnanediol remaining.

WORK ON RABBITS

This particular work was undertaken to ascertain:

1. If rabbits excrete Na pregnanediol during pregnancy.
2. If so, to follow the variations in concentration during the gestation period.

Six individual metal cages were fitted out so that the urine from each cage would drain into a beaker set beneath.

The rabbit colony (13 females and 3 males) was examined for females which might be in estrus. Three which seemed likely were placed with the bucks on April 28, 1938, and two more on April 29. All five were placed in collecting cages on April 30.

Owing to unforseen difficulties it was impossible to analyse any samples before May 10. None of the samples collected from April 30 to May 10 gave any trace of combined pregnanediol. All subsequent samples until the end of the experiment, when the litters arrived on May 29 and May 30 were negative. All five rabbits had been pregnant.

To check the possibility that the combined pregnanediol had been completely hydrolysed before analysis, it was decided to repeat the experiment.

On September 6, the six most likely does were selected from the colony. Two bucks were placed with them (3 does and a buck to a cage), and the bucks interchanged between cages daily until Sepbember 9. At this time the does were placed in the collecting cages.

Samples were collected from each cage daily, pooled, and an aliquot analysed on the day collected. The samples for the first ten days were negative. Samples were thereafter collected from each rabbit independently and in sequence, until the normal gestation period was over. In one cage, once 3 mg. of a substance giving a very poor melting point, about 220°C was noted. All the samples were negative. At the end of the normal 30 day gestation period, it was obvious that none of the animals had been pregnant.

From these two experiments, it can be seen that there are no substances in normal rabbit urine which would precipitate in a Venning analysis. From the complete blanks of the first experiment, it seems

likely that if rabbits excrete progesterone, they do not excrete it as Na pregnanediol glucuronidate.

THE ISOLATION OF FREE PREGNANEDIOL

Dr. Weil's procedure was received during the first experiment with rabbits, described earlier. As a check to ascertain if negative results were being obtained because the pregnanediol was not present in the combined form, 2500 cc. of the final week's samples was saved for the Weil procedure.

The residue from the butanol extraction was taken up in 30 cc. acetone and 30 cc. N/10 NaOH, 90 cc. NaOH was then added slowly with heating. When this was centrifuged, the clear liquid was saved for further checking. The residue was treated with acetone, and NaOH according to instructions, but would not dissolve. The mixture was then boiled to remove the acetone, and extracted again with butanol as in the second extraction of the Venning procedure. The extract was evaporated to dryness in vacuo, and again treated by the Weil procedure. No crude pregnanediol separated out, nor could any trace of it be found.

The clear liquid from the first centrifuging was brought back to neutrality with 2 N HCl. A

tarry mass separated out and was removed by centrifuging and filtering. It was dissolved in acetone, boiled with charcoal and filtered. An attempt to precipitate this by the Weil method brought only a little tar.

The clear filtrate was made strongly alkaline. A substance somewhat resembling ferrous hydroxide precipitated. It did not contain iron, but was soluble in acid, insoluble in alkali, and all organic solvents tried. No organic matter could be extracted from it by alcohol, acetone or ether. It was therefore concluded that there was no pregnanediol present.

To summarize this, it may be said that an attempt to apply the Weil procedure to the urine of pregnant rabbits showed considerable interfering substances, but no trace of pregnanediol.

The complete failure to demonstrate combined pregnanediol by the Venning procedure, or free pregnanediol by the Weil procedure, naturally suggested checking back on the experimental procedures. The Venning method had previously been tried satisfactorily on human pregnancy urine.

To check the Weil procedure, a sample of humah pregnancy urine was obtained from Dr. Charlesworth. Four 300 cc. portions were taken as soon as the sample was received.

The first two samples were treated as controls, by the Venning procedure, with one modification. Weil suggests only two extractions, with 50 and 25 cc. of butanol, instead of the four extractions recommended by Venning. The Weil extraction was followed for all four to make conditions more comparable. In the first two portions, 14 and 15 mg of combined pregnanediol were found by the Venning method. This represents about 9 mg. free pregnanediol for a 300 cc. sample. The precipitates darkened at 240°C, but melted with decomposition at 268°C. This indicates a fairly pure precipitate.

When the acetone concentrates from the second two portions were diluted with NaOH in the Weil procedures, it was found impossible to precipitate slowly enough to avoid an immediate precipitate. The precipitate was of colloidal dimensions, and no method was found to aggluntinate it sufficiently to make it possible either to centrifuge or filter.

As soon as the NaOH was added, the solution became a homogeneous white colloidal solution, and would remain that way for several weeks. The addition of acid apparently caused agglutination. However, centrifuging or filtration served only to re-emulsify the material. The addition of a small amount of ether completely destroyed the colloidal state. No pregnanediol was isolated from these samples.

This work indicates that the Weil method , as received, does not work. Dr. Cantor of the Department of Biochemistry, also wrote to Dr. Weil for his procedure. He obtained results with it, similar to those outlined above. Apparently the Weil procedure could not be used, but as it was still desirable to be able to isolate free pregnanediol, many modifications were tried.

An interesting sidelight was thrown on the Weil procedure in some later work. Fairly pure crystalline pregnanediol, prepared by a method described later, was dissolved in acetone, and precipitated by the addition of warm NaOH. This was centrifuged, the precipitate dissolved in alcohol and precipitated by water. The precipitate was re-

dissolved in alcohol, centrifuged, and the clear supernatant liquid poured off, and evaporated to dryness. The result was a sample of very pure pregnanediol, melting sharply at 235°C. This is very interesting, as it shows that working with pure pregnanediol, Weil's system of precipitation from organic solvents by NaOH and water, is a sound one. However, in every case in which his procedure was tried on urine, interfering substances in the urine prevented the proper precipitation of pregnanediol.

Before describing further experiments on the isolation of pure pregnanediol, it seems desirable to outline the properties of pregnanediol as described by Marrian. (16.)

Crystallizes as thin, hexagonal plates, melting sharply at 232-5° (uncor.), and without decomposition.

Completely insoluble in hot or cold water, or in dilute aquaeous acids or alkalis.

Very slightly soluble in ether, ligroin and cold acetone.

Solubility greater, but still less than 1% in cold benzene, toluene, ethanol, methanol, and chloroform.

Boiling acetone is a good solvent, the best for crystallizing.

Three more portions of the same sample, W 3, W 4, and W 5, were incubated to hydrolyse the combined pregnanediol.

W 3. The residue after extracting the butanol extracts to dryness, was extracted several times with hot ether, and hot acetone. The material remaining in the flask was completely soluble in aqueous alkali, and therefore did not contain pregnanediol. An attempt was made to separate pregnanediol from the ether and acetone solution, but without success.

W 4. Residue after evaporating butanol extracts to dryness taken up in hot alkali. Cooled and centrifuged. Some solids floated, while others sank. The light solids were filtered off (some loss by emulsification) and extracted with boiling acetone. On cooling, a small amount of white material settled out. This melted at 235° C and was probably pregnanediol.

W 5. An attempt was made to substitute ether for butanol in the original extraction. This was unsatisfactory, due to the formation of

large

quantities of a jell. This was much more difficult to handle than the butanol, and a change to ether was therefore not advantageous.

On July 29, two new samples of urine were obtained from Dr. Charlesworth. One Venning procedure was run on a portion of each, giving 28 mg. and 33 mg. to 300 cc. A third portion was made acid with 5 cc. conc. HCl before extracting. This was done as a result of several observations that emulsions of solid and butyl alcohol formed during extraction, broke up upon the addition of acid. However, acid apparently made numerous tarry constituents less soluble in the aqueous fraction. In consequence, the butanol extract contained so much tar as to be unfit to continue with the procedure.

All later samples were extracted without the addition of either acid or alkali, as originally advocated by Venning. Incubation almost invariably brings about an alkaline reaction anyway, the condition advocated by Weil.

In the following runs, each portion was 300 cc., and was incubated for at least 24 hours, and

usually longer, and extracted with 50, 25, and 15 cc. portions of butanol. The extracts were centrifuged, and the clear alcohol evaporated to dryness. The differences are all subsequent in treatment of the residue.

W 6. Residue taken up in 10 cc. 2 N NaOH. Flask washed twice with 5 cc. portions of 2N NaOH. Extract and washings contained much suspended matter. Centrifuged. A white apparently amorphous solid, lighter than the solution separated out. Filtered by gravity. The flask and centrifuge tube were rinsed with water. The solid which had gone to the bottom of the tube was ignored, as pregnanediol was known to be quite light. The warm alkali broke up the filter paper. No pregnanediol was separated from the mixture of filter paper, NaOH, and other impurities.

W 7. Residue taken up in 2N NaOH as before. Alkaline solution extracted with 10 cc. portions of ether until it cleared. Considerable solid separated out of the ether, and centrifuging brought it to the ether-water interface. This was filtered and washed with water. Some of the solid came

through as shown by further centrifuging of the filtrate. Material on filter paper leached with EtOH and evaporated to dryness. Small amount of tarry material remained. Clear ether filtrate did not contain anything of interest.

A precipitate appeared later in the original alkaline solution. This was centrifuged off and washed once with water, in which some of it dissolved. The remainder was boiled with acetone. The portion which would not dissolve was centrifuged off, and proved to be completely soluble in water. Therefore it was not pregnanediol. The acetone was evaporated to dryness, leaving a yellow solid, melting gradually between 100° and 240° C. This was probably impure pregnanediol.

W 8. Residue taken up in 2N NaOH as before. The suspension was warmed and extracted with warm benzene. Jells formed which were not broken up, even by centrifuging. The benzene and extracts were made acid, and extraction was then easy. The benzene gave only a tarry liquid. Other solids in original solution and wash waters were extracted with acetone, but no pregnanediol was obtained.

W 9. Residue taken up in 2N NaOH. The flask

was rinsed twice with 2 cc. portions of 2N HCl. This extract was left standing over the week-end, Centrifuged, and all of the solid collected at bottom of tube. Solid shaken up with N/10 NaOH and again centrifuged. Much of the colored material dissolved, leaving a whiter more gelatinous mass. This was shaken up in water with enough HCl to make acid. Centrifuged, and solid formed a cake on top of tube. Liquid poured off and solid shaken up with distilled water. Centrifuged, and solid again on top. Filtered and dried. Filter paper leached with boiling acetone. A small amount of material melting at 225° was separated from the acetone.

W 10 and 11. Residue taken up as in W 9. Centrifuged, and clear supernatant liquid poured off. Residue shaken up with 5 cc. 1N NaOH and heated in a water bath. 5 cc. H_2O added to tube, centrifuged, and clear liquid poured off. Residue now white and gelatinous, washed with H_2O. Acetone was added to the residue which dissolved completely. Centrifuged to remove suspended dust. W 10 evaporated to dryness, leaving a waxy solid which melted and gave off pungent fumes on further heating.

W 11 evaporated almost to dryness, and then cooled. An impure concentrate separated out, melting at 140°.

Attempts to isolate pregnanediol from various wash waters and filtrates of W 9, 10 and 11 were completely unsuccessful. Combined pregnanediol was also tested for by the Venning procedure and not found.

W 12. Taken up in 10 cc. hot 2N NaOH, and flask washed twice with 5 cc. of the same. This was allowed to cool, and then centrifuged. Layers of solid on top and bottom. Top solid, and some of solution poured into another centrifuge tube and diluted with water. Centrifuged, and because of decreased density of solution, solid went to the bottom. Residue washed with 2N HCl and then water. The remainder dissolved completely in acetone. Centrifuged, and evaporated on a water bath. Solid was tarry and melted about 140 C. Washed again with conc HCl and water. Filtered and precipitate leached with hot acetone. Acetone evaporated, and precipitate was still tarry. Precipitate in beaker rinsed twice with cold ether, leaving a white solid melting at 225° C probably pregnanediol.

W 13. Taken up in 10 cc. hot 2N NaOH. Flask rinsed twice with 5 cc. 2N HCl. Left over night and centrifuged. All solid remained at bottom of tube. Solid stirred up in 5 cc. 2N NaOH, and digested 10 minutes in a water bath, to dissolve as much tar as possible. Centrifuged, and digested with 5 cc. water. Centrifuged, and dissolved in hot acetone. Put in weighed beaker and evaporated to dryness. Solid in beaker rinsed three times with cold ether. The material remaining weighed 13 mg. (20 mg. Calculated from Venning assay of sample) and was too tarry for a melting point.

Apparently all samples which have acid added near the beginning of the experiment show excessive precipitation of tar. Washing with NaOH apparently dissolves tar, but too strongly alkaline solutions are so dense that pregnanediol and other materials associated with it, tend to float.

W 15 & 16. Residue taken up in 5 cc. 2N NaOH to which 5 cc. water was added after sample seemed to have gone into solution. Flask was rinsed with two 5 cc. portions of water. Centrifuged.

Considerable solid separated out, but a small amount remained suspended in supernatant liquid of each tube. This was filtered off and shown to be mainly impurity with traces of pregnanediol. To the residue in the centrifuge tubes, 2 cc. of 2N NaOH were added and digested in a water bath. Each tube made up to about 5 cc. with water, and centrifuged. NaOH solution poured off (W 15 and 16 joined for convenience at this point). Washed with 5 cc. water and then digested twice with 5 cc. HCl. Finally washed with water and then dissolved in 5 cc. EtOH. Heated to complete solution, centrifuged and clear liquid poured into a clean beaker. Evaporated almost to dryness and left over-night. Crystals grew from all sides of beaker (see figures 7 & 8.) Traces of mother liquor left around crystals washed out with ether. This, on evaporation, gave only tar. Redissolved and water added gave colloidal solutions similar to those obtained in Weil procedure. A few crystals were lost mechanically. Remainder weighed 17 mg. (theoret. 19) and melted at 225° C (theoret. 235°.) This was the material mentioned earlier, used to check Weil's precipitation method. The result was pure

Figure 7.

Pregnanediol crystals from alcohol in 50 cc. beaker.

Figure 8.

Same crystals as Figure 7. X50

Figure 9.

Pregnanediol crystals from acetone. X100

Figure 10.

Unknown inorganic crystals from dog urine. X50

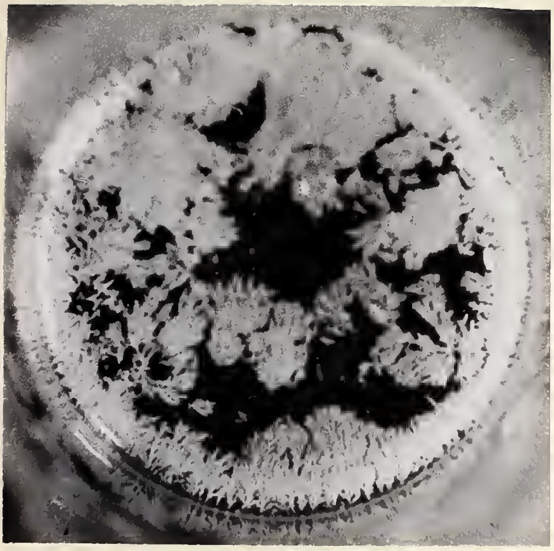


FIG. 7.

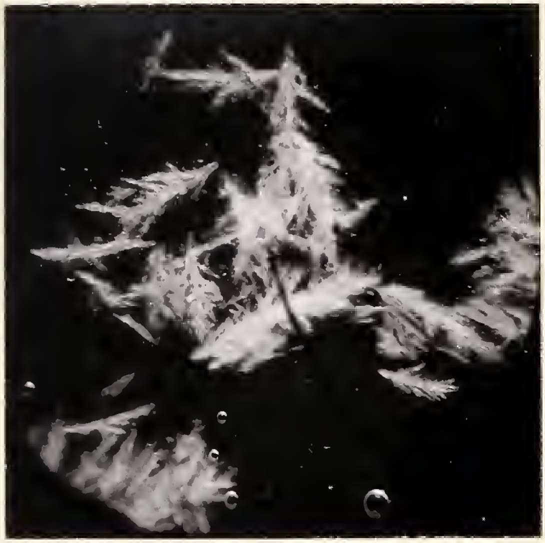


FIG. 8.

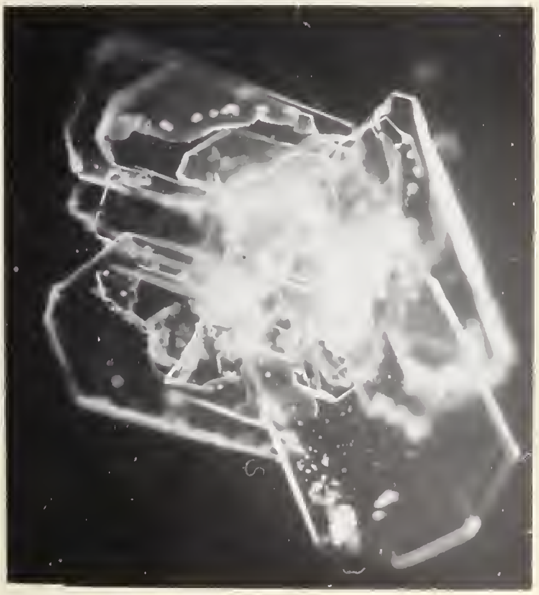


FIG. 9.

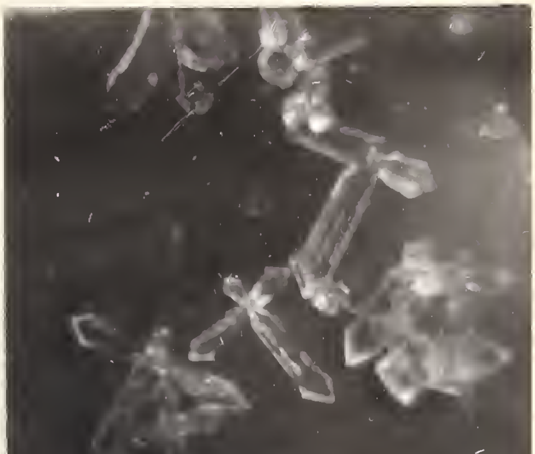


FIG. 10.

pregnanediol. When crystallized very slowly from acetone, it was possible to get crystals (fig. 9.) similar to those described by Marrian. (16.) These crystals belong to the ortho rhombic system.

W 17. A slightly larger sample, treated as in W 15 and 16. A good yield was obtained, but the procedure could not be considered quantitative.

The main difficulty seemed to be in getting complete precipitation in the centrifuge tubes. A faster centrifuge might have cured this, but was not readily available.

At this time, two new samples of urine were obtained from Dr. Charlesworth. Each was assayed immediately by the Venning procedure, and both gave 11 mg. of combined pregnanediol in 300 cc. portion. This is roughly equivalent to 22 mg. per litre of pure pregnanediol.

W 18 and 19. In view of the recent success of the Weil procedure on pure pregnanediol, it was decided to give it a final chance. Once more, colloidal solutions, which could not be filtered or centrifuged, were obtained. They were eventually discarded.

W 20 and 21. Instead of 5 cc. 2N NaOH, and three 5 cc. portions of water, W 20 was taken up in four 5 cc. portions of N/10 NaOH. W 21 in the old way. W 21 gave a coarser sediment, but the same difficulty was experienced in getting a complete sedimentation. To aid in complete precipitation, a small amount of $Al\ Cl_3$ solution was added to the tubes, and the pH adjusted to bring down the $Al\ (OH)_3$. This brought about a complete sedimentation, the pregnanediol apparently being caught and carried down by the thick jelly-like precipitate. Pure pregnanediol was isolated but not quantitatively. It was decided to scrap aluminum in favor of iron, due to the greater stability of the hydroxide in alkaline solution, also because iron remaining as an impurity is easier to detect.

W 23. As pregnanediol is insoluble in water, it was decided to try to ascertain if all the pregnanediol was in the sediment which invariably appears after incubation.

Part of sample was filtered by gravity. The other half was filtered by suction along with ferric hydroxide. Apparently gravity filtration (very slow)

or centrifuging (with or without $Fe(OH)_3$) are the only ways to separate sediment.

Pregnanediol was isolated from the sediment, identified by melting point 225° C and hexagonal crystals from acetone.

The clear filtrate was extracted as in W 14, and again pregnanediol was isolated. This experiment was not done quantitatively, and was not repeated but it indicated that the original extraction could not be dispensed with.

W 24. Extracted as usual and centrifuged with $Fe(OH)_3$. Washed with NaOH twice, dissolving and reprecipitating the $Fe(OH)_3$ between centrifugings. Washed with water. The hydroxide precipitate was then extracted with alcohol, and the alcohol evaporated. This gave crude pregnanediol.

This work was discontinued at this point, to go on with the animal work, but the following comments seem pertinent towards making a quantitative procedure for pregnanediol.

The use of an hydroxide for separations precludes washing with acid, and is apt to leave a certain amount of urea and other acid soluble

impurities behind. Secondly, the final rinsing with ether seems rather crude.

A possible method would be to dissolve $Fe(OH)_3$ in a cold solution of HCl in acetone and centrifuge. The crude pregnanediol should not dissolve to any great extent, and after another washing with a small amount of cold acetone, it should be possible to centrifuge the precipitate with ether, without having the tarry pregnanediol protected from the ether at the bottom of an aqueous layer. The residue might then be dissolved in alcohol and filtered into a weighed vessel.

Before leaving the subject of human urine, mention might be made of certain other substances noted. In numerous samples, after incubation, very fine crystals were seen at the bottom. Several batches were separated out and examined. None of them melted even when heated to redness, so were obviously not organic. By reference to standard works on clinical microscopy, they were identified as $CaHPO_4$ and $Mg\ NH_4\ PO_4$. Some of the magnesium ammonium phosphate crystals bore a strong superficial resemblance to pregnanediol crystals.

Under somewhat similar conditions, crystals were separated from dog urine. These are shown in figure 10. They were shown to be definitely inorganic, but have not been identified.

WORK ON DOGS

The work on rabbits during September, showed that it would probably be a waste of time to try breeding them again before spring. It was clear that some other experimental animal would have to be used for animal work during the winter.

On November 30 a female dog, supposedly pregnant, was received. It was placed immediately in a collecting cage loaned by Dr. Hunter. Between November 30 and December 7, five samples were taken On December 5, she had eight pups.

Aliquots (300 cc.) of all samples were extracted by the Venning procedure immediately after removal from the cage. Instead of the fine, white crystalline precipitate of combined pregnanediol obtained with human urine, a thick gelatinous precipitate came down with dog urine. The amount of this material fell off markedly at the time the pups came. The solids, when separated out, were obviously very impure. No melting point could be obtained. The first precipitate was warmed with 2 cc. water and acetone added to make 40 cc. On cooling, the residue had lost about two thirds of

its weight, and was still tarry. This observation naturally suggested loss in the acetone used to precipitate the other samples. The acetone had been saved, and was pooled and evaporated to dryness. There was a large crystalline residue, which was worked over, yielding considerable amounts of crystalline urea and a little tar.

The precipitates from the last four samples were combined for an attempt at purification. The material was found to be very soluble in hot alcohol. After four re-crystallizations from alcohol, the material seemed fairly pure, although still gelatinous. It melted with decomposition between 200° and 215° C.

The urine saved out during the short period available, was incubated at 37° C for 24 hours. The extract was centrifuged, evaporated to dryness and taken up in 20 cc. N/10 NaOH. This was extracted three times with butanol, 10, 5, and 5 cc. amounts being used. The butanol was washed with three 2 cc. portions of 2N NaOH, two 2 cc. portions of 2N HCl, and water. The butanol was then evaporated to dryness. The residue was taken up in a mixture of acetone and ether, and evaporated almost to dry-

ness. It was put out in -20° to -40° F. overnight and filtered in the morning. A solid separated out, melting between 48° and 52° C, without decomposition. More of the same material was separated from earlier filtrates etc. in this run.

A second dog, reported to be about one month pregnant was received on January 25. She was placed in the collecting cage immediately. During the 45 days, during which samples were taken, she averaged approximately only 170 cc. of urine per day. For this reason, it was not possible to collect samples every day. Every sample collected was used completely for extraction by the Venning process. The amounts of urine and precipitate obtained will be seen in Table 1. The date given is a mean date for the period represented by the sample, and obtained by assuming the pups were born on the 63rd. day of the gestation period and calculating back. Samples 1 to 5 inclusive represent the first dog. Only the first was weighed, due to their obvious impurity.

Residues from samples 7, 8, and 9 were used in an attempt to re-crystallize from methanol.

Table I

Sample No.	Mean Date	Time in Hours	Volume in cc.	Total Solids mg.
1	58	27.5	280	22.2
6	26	44.5	500	5.7
7	29	123	1120	219.1
8	33	46	525	78.6
9	36	122	825	136.9
10	40	46	365	149.3
11	43	77	1140	84.2
12	48	164	680	25.9
13	54	94.5	1055	26.2
14	57	98.5	620	77.1
15	62	72.0	550	20.3
16	64	46	380	19.5
17	68	119	475	14.2
18	72	75	530	15.5

This was attempted because of a similarity of properties between the solid obtained and Na estradiol glucuronidate, when precipitated from alcohol. From methanol this latter material gives a crystalline product melting at 303° C. No such result was obtained. An attempt was then made to hydrolyse any esters which might be present to liberate free sterols if any. Nothing but ammonium chloride and tar was isolated.

With samples 10-18 inclusive, an attempt was made to separate the solid material obtained by the Venning procedure into the fraction melting around 200°C and anything else present. The method used was worked out theoretically from properties observed in samples 2 to 5 and appeared to work fairly satisfactorily.

A small amount of 95% alcohol was added to the precipitate in the beakers and heated on the water bath until all solid had dissolved. This solution was then evaporated down to one or two cc. depending on the amount of solid originally present. The beaker was removed from the water bath, and 2 cc. of chilled ether added with stirring, and the beaker set in snow to cool. This was centrifuged

in a 5 cc. centrifuge tube, and the solid washed once with cold ether. This seemed to give a fairly clear separation of ether-soluble and ether-insoluble substances. In a few cases, it was possible to get a small second crop of material. The amounts of the two fractions are shown in the table 2.

The ether soluble substances were pooled and carefully fractionated. A clear non-crystalline waxy material melting at about 50° C and urea were obtained. These materials did not appear to be specific to pregnancy. The waxy material resembled part of tarry impurities met with in work on human urine.

In Fig. 11 is plotted the concentrations of the original precipitate, and the higher melting point material. For comparison and reduced to the same time scale, is a curve published by Wilson and Randall (4) for concentrations of pregnanediol during pregnancy in humans, worked out by the Venning procedure. It might be noted that their comparatively smooth curve was obtained by averaging 79 determinations. Individual variations for the eighth month ranged from 18 to 126 mgs. per day.

Table 2

Sample No.	Mean Date	Total Solids/ 24 hrs.	E. Sol./ 24 hrs. mg.	E.Insol./ 24 hrs. mg.
10	40	78.0	22.0	53.5
11	43	26.2	11.7	14.7
12	48	3.8	2.5	1.27
13	54	6.7	5.2	1.4
14	57	18.8	10.9	7.8
15	62	6.8	4.1	2.7
16	64	10.2	7.4	2.9
17	68	2.9	2.9	0
18	72	5.0	5.0	0

The ether insoluble fraction was further purified by pouring an alcohol solution into cold ether, and cold chloroform. The purest fraction was obtained by diluting a benzene solution with ligroin and cooling. This gave material which seemed quite pure and melted at 230°C. It did not appear crystalline under the microscope, but showed some of the refractile characters of crystalline material between crossed Nicols.

Some of this material was refluxed for half an hour with alcoholic NaOH. The hydrolysate was extracted with benzene. The benzene was evaporated leaving a crystalline mass. This was further purified by precipitating from ether with ligroin. This gave pure crystalline material melting at 52°C. Note a similar melting point to substance obtained earlier from dog pregnancy urine by free pregnanediol procedure.

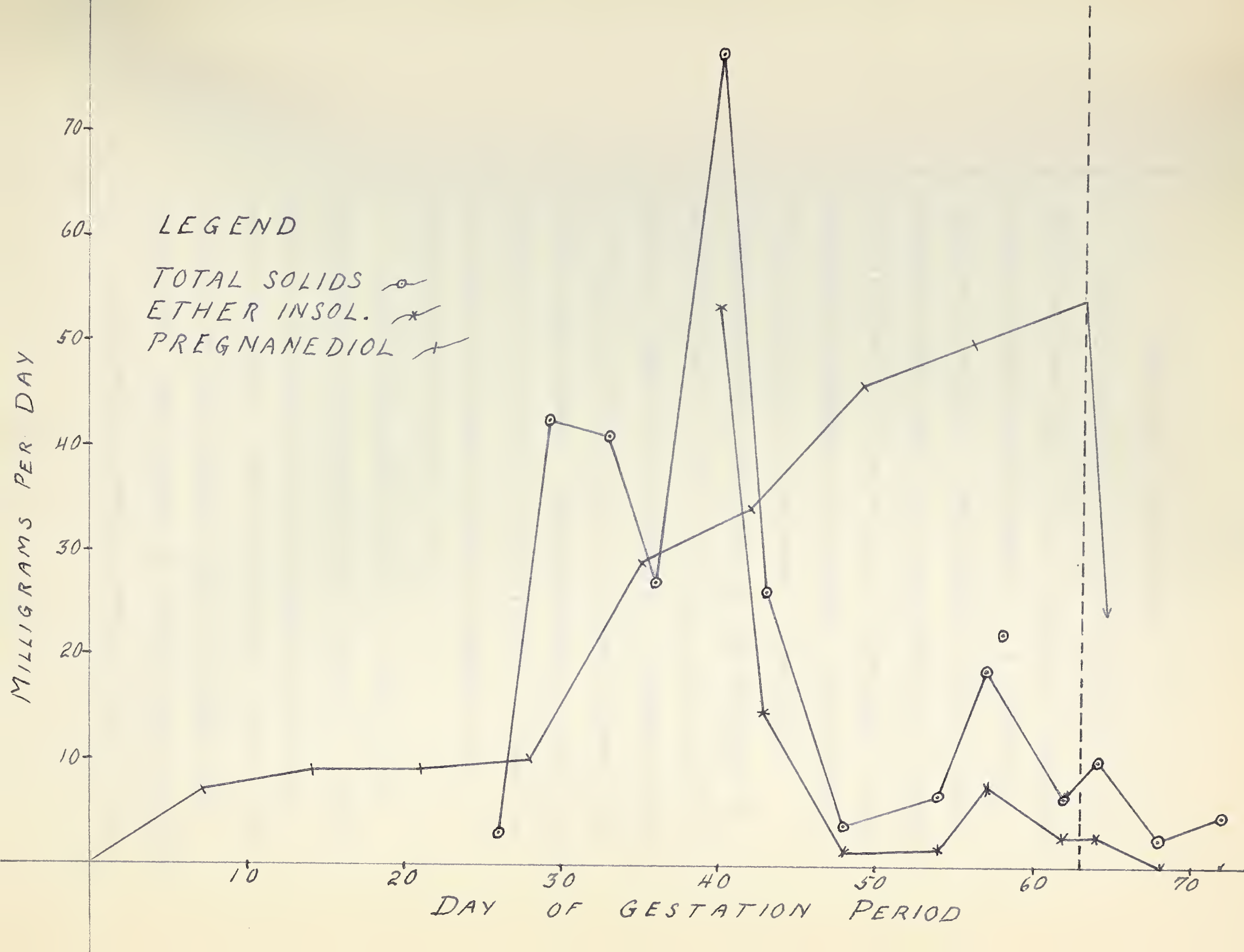
This substance is very soluble in all organic solvents tried.

Larger quantities of these two compounds must be obtained before their composition can be worked out.

Figure 11.

Graph showing variations in concentration during the gestation period of,--

1. Total solids obtained by the Venning procedure from dog urine.
2. The ether insoluble fraction of the above.
3. The pregnanediol concentration for human pregnancy as reported by Wilson and Randall, This is reproduce d for comparison, reduced to the same time scale, i.e. 63 days for a dog equals 9 months for humans.


LEGEND
TOTAL SOLIDS
ETHER INSOL.
PREGNANEDIOL
MILLIGRAMS PER DAY
70
60
50
40
30
20
10
10
20
30
40
50
60
70
DAY OF GESTATION PERIOD

SUMMARY AND CONCLUSIONS

In the endocrine research reported so far, there has been very little evidence of any specificity of particular hormones to a particular species of animal. For example, the follicular hormones were found to be the same in all animals investigated, with exception of the horse, which apparently produces, in addition, other closely related estrogenic substances. Similarly, progesterone has been found in the extracts of corpora lutea of certain animals, and assayed on others. Therefore, by analogy, it seems natural to expect various animals to excrete progesterone in the same manner. The experimental results here reported do not seem to justify that assumption. An attempt will now be made to evaluate the significance of those results.

In the work with rabbits, no trace of Na pregnanediol glucuronidate was observed. Unfortunately, the possibility of complete hydrolysis, cannot be denied, but from Venning's work on the stability of the compound (22.), it seems very improbable. The attempts to isolate free pregnanediol by the Weil procedure, were of little value,

and can only justify the rather negative statement that no pregnanediol was found. There is no satisfactory evidence that it was not present. From the work on the biological assay of progesterone done on rabbits, it seems reasonably certain that they produce progesterone during pregnancy, but from the present work, they do not seem to excrete that PROGESTERONE ~~pregnanediol~~ as Na pregnanediol glucuronidate as was expected.

From numerous trials, the Weil procedure for the estimation of free pregnanediol in urine, seems to be useless. This failure was apparently caused by interfering substances present in the urine, not by anything theoretically unsound in methods of extraction and precipitation.

Free pregnanediol was isolated from human urine by new procedures. Much useful information was obtained about the requisites of such a procedure, but difficulties in making a complete separation, make it impossible to claim a quantitative procedure.

The determination of pregnanediol is getting to be of considerable importance clinically. In cases of habitual abortion, the concentration of

pregnanediol is low, and the administration of progesterone can often be used in such cases properly to maintain pregnancy. Recently, the Mayo Clinic (4) has used the Venning procedure as a test for early pregnancy when other tests seemed doubtful. They apparently found the test valuable for this. As the possibility of hydrolysis is always present for this method, it is obvious that a good method for determining free pregnanediol would be very valuable as the best method of following the function of the corpus luteum and other progesterone forming bodies during pregnancy.

With dog urine, it was impossible to demonstrate either free or combined pregnanediol. In this case, the question of hydrolysis need not be considered. Similarly, while a complete separation of free pregnanediol could not have been expected, if any had been present, some traces would have been found. Thus we can say, fairly safely, that during the second half of canine pregnancy, no pregnanediol is excreted in the urine. This is not entirely without precedent. In 1934, Marrian et al. (24) looked for pregnanediol in the urine of pregnant mares. They were unable to find any, but did find a new

solid alcohol, with one more hydroxyl group than pregnanediol.

Two unknown compounds were found in the urine of both dogs used. One, that obtained by the Venning procedure, gave the other on alkaline hydrolysis. The second compound was also found when looking for free pregnanediol. These substances appeared to be specific to pregnancy, and a curve was plotted of the variations in concentration during the gestation period. The highly irregular nature of the "curve" obtained must be attributed to the uncontrollable errors in such work. The completeness with which the urine is collected will vary considerably, and uncontrollably. The amount and composition of the various samples must also affect the results, particularly as the procedure used was not developed for this substance. Speculation as to the significance of these two substances is of little use until their composition is known. It is to be hoped, of course, that they will prove to be excretion products of progesterone.

In conclusion, the author wishes to express his gratitude to the Department of Physiology and Pharmacology, both for encouragement and for placing

the facilities of the department at his disposal during this research. The author also wishes to acknowledge gratefully the loan of a large animal cage by Dr. Hunter, useful advice and assistance from Dr. Cantor, and the provision of test material by Dr. Charlesworth.

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